

FUNCTIONAL AND STRUCTURAL ROLE OF  
ARGININE 103 IN HUMAN ERYTHROPOIETIN

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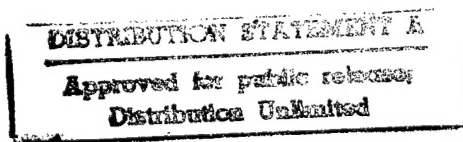
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## SUMMARY

The identification of amino acid residues essential for function of the hematopoietic growth factor erythropoietin has been approached by several methods, including comparisons of related sequences, immunochemical approaches, mutagenesis and computer modeling. We have reported previously that mutations within amino acids 100-109 of erythropoietin can have profound effects on the hormone's structure and/or activity and that Arg103 is especially important for function (Chern, Y., Chung, T. & Sytkowski, A.J. (1991) *Eur. J. Biochem.* 202, 225-229; Grodberg, J. Davis, K. L. & Sytkowski, A. J. (1993) *Eur. J. Biochem.* (218, 597-601). We have now constructed a series of Arg103 substitutions in order to determine the structural features of amino acid 103 required for biological activity. Each of the mutants was expressed and secreted efficiently by transfected COS1 cells. Mutants Arg103Asn, Arg103Gln, Arg103Glu exhibited no biological activity. In contrast, Arg103His and Arg103Lys had specific activities equal to 2 % and 25 %, respectively, that of wild-type erythropoietin, respectively, indicating that a positive charge may be required at position 103 but that other constraints necessitate the presence of Arg for full activity. A role for amino acid 103

in the protein's structure was supported by the results of experiments which revealed marked differences in heat stability among the mutants. We hypothesize that an Arg at position 103 may confer sufficient flexibility to the receptor binding domain to facilitate initial binding to the receptor and may then stabilize the binary complex by hydrogen bonding with carbonyls of the receptor protein.

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## INTRODUCTION

Attempts to determine the three-dimensional structure of the hematopoietic growth factor erythropoietin (Epo) have met with little success to date. The length of the polypeptide chain (165 aa.) precludes analysis by currently available NMR techniques. Furthermore, the high carbohydrate content of the glycoprotein (approx. 40% by weight) has interfered with attempts at crystallization. Although the deglycosylated hormone remains biologically active, it forms complex aggregates, thereby closing this avenue for obtaining meaningful x-ray diffraction patterns.

Several alternative approaches have been employed to elucidate Epo's structural and functional properties. Specifically, the methods used to identify those amino acid residues essential for the Epo/receptor interaction include: 1) the analysis of conserved sequences among mammalian Epos (1, 2); 2) immunochemical studies with site-specific antipeptide and/or monoclonal antibodies (3-10); 3) mutant studies by means of deletion, linker-scanning, substitution and alanine scanning mutagenesis (11-15); 4) computer modeling (11,14,16,17). While each approach has provided useful

information, a model structure that can reconcile all of the experimental data has not yet emerged.

In a previous series of immunochemical and mutagenesis-based studies, we identified amino acids 99-110 of the mature human Epo protein as forming part of a region of the molecule critical for both structure and function (5,12). Recently, we performed alanine-scanning mutagenesis and determined that the activities of mutants Ser104Ala, Leu105Ala and Leu108Ala were reduced to 16, 44 and 37% of wild-type Epo, respectively, while Gly101Ala was increased to 130% (13). Most importantly Arg103Ala was completely inactive, even though immunochemical and thermal denaturation studies showed no significant overall structural alteration or destabilization. Another substitution, Arg103Asp, was also inactive, but was markedly more stable than wild-type. These results suggested a critical role for Arg103 in receptor recognition.

We have now substituted Arg103 with a series of charged and/or polar residues in order to determine the structural features of amino acid 103 required for biological activity. The results demonstrate that full biological activity is observed only with Arg at position 103. Moreover, the

data strongly suggest that another positively charged residue lies in close proximity to Arg103 in three-dimensional space and that interaction (or lack of interaction) between amino acid 103 and the other positively charged residue may have a major influence on the protein's structural stability.

## MATERIALS AND METHODS

### Construction of Epo variants

pSelect-Epo served as the starting material for all mutagenesis reactions (12,15). Single-stranded DNA isolation and all subsequent mutagenesis steps were performed as described in the Altered Sites Mutagenesis Kit manual (Promega Corporation). Oligonucleotides were purchased from Genosys Biotechnologies, Inc. Each oligonucleotide cointroduced a specific codon change and a restriction endonuclease site (*Hinf*I) to facilitate screening for the desired mutant. Individual colonies arising from the mutagenesis reaction were purified, and their plasmids were

isolated by the alkaline-lysis procedure (18). Plasmids were restricted with the appropriate enzyme (New England Biolabs) and the digests were fractionated on 1.7% agarose gels. The DNA was transferred to GeneScreen Plus (DuPont) and hybridized with a <sup>32</sup>P-labeled *Eco*RI fragment encoding Epo(12). Clones containing the new restriction sites were sequenced by the dideoxynucleotide method (19) to verify the presence of the desired mutation and to confirm the sequence fidelity of the entire construct. The *Kpn*I-*Bgl*II subfragment of each mutant was moved into pSV2-Epo (12) from which the corresponding wild-type sequences had been removed by restriction digestion. These constructs were used in all subsequent experiments.

### **Cell culture and transfections**

COS 1 cells (ATCC) were maintained in Dulbecco's modified Eagles' medium containing 10% fetal bovine serum (Gibco-BRL Life Technologies). Cells grown to 40-60% confluence were transfected with 5 µg DNA, using DEAE-dextran and 0.1 mM chloroquine (20). Medium was collected 3 days post-transfection and centrifuged at 13,776 x g for 10 min to remove cell debris.

The biological activity of each sample was determined by the Krystal *in vitro* bioassay (21). Epo protein concentration was measured by competitive radioimmunoassay (RIA), calibrated against pure, recombinant human erythropoietin (INCSStar).

### **Heat-stability assay**

The heat stability of each mutant Epo molecule was evaluated as described previously (13). Briefly, aliquots of conditioned medium from transfected COS cells were incubated at 56°C for specified time intervals. Samples were cooled on ice, then evaluated for structural integrity/denaturation by a conformational sensitive radioimmunoassay. Results are given in terms of the percentage of protein immunoprecipitated after heat treatment compared to untreated samples.

## RESULTS

### Construction and biological activity of erythropoietin mutants

Our previous experiments indicated that substitution of Arg103 of wild-type recombinant human Epo with Ala abolished biological activity, whereas substitution of Arg103 with the negatively charged Asp abolished biological activity and enhanced structural stability markedly (13). Therefore, several amino acid substitutions were made at the position 103 of erythropoietin by site-directed mutagenesis in order to elucidate further the role of this Arg residue in the hormone's biological activity and structural stability. Besides the Arg103Ala and Arg103Asp mutants described previously, the following mutants were prepared: Arg103Asn, Arg103Gln, Arg103Glu, Arg103His and Arg103Lys. The oligonucleotides used for the mutagenesis reactions are shown in Figure 1. Each oligonucleotide was designed to replace Arg103 with an alternative residue and simultaneously to introduce a restriction endonuclease site (*Hinf*I) to facilitate screening. Clones identified by restriction analysis were sequenced to confirm sequence integrity and were transferred to the

eukaryotic expression vector pSV2 (12). These constructs were introduced subsequently into COS cells. After 72 hours, the transfected culture supernatants were harvested and tested for biological activity by in vitro bioassay. Epo protein concentrations were determined by radioimmunoassay.

All Epo mutants were synthesized and secreted efficiently by transfected cells (Table 1). Expression levels comparable to that seen for wild-type Epo were observed, indicating that none of the amino acid substitutions altered protein conformation drastically or affected transport. This finding agrees with our previous results for Arg103 substitution mutants (13) and contrasts with results obtained in linker-scanning mutagenesis experiments, which showed that replacement of groups of three or four amino acids with Glu-Phe interfered with proper protein folding and/or secretion (12).

The specific activities of the mutant Epo molecules relative to wild-type Epo are shown in Figure 2. Interestingly, two substitutions, Arg103His and Arg103Lys, resulted in recombinant proteins with specific activities equal to 2% and 25% that of wild-type protein, respectively. None

of the other amino acid replacements generated proteins with detectable biological activity.

### **Heat stability of wild-type versus mutant erythropoietins**

Wild-type Epo exhibits a time-dependent decrease in biological activity when incubated at 56°C or above (22). We demonstrated previously (13) that a concomitant change in protein conformation of both biologically active (wild-type) and inactive Epo (Arg103Ala, Arg103Asp) could be monitored by radioimmunoassay which recognizes one or more heat-sensitive epitopes. These previous experiments showed that the stability of Arg103Ala was virtually identical to that of wild-type Epo, whereas the stability of Arg103Asp was much greater (13). Therefore, we carried out heat stability assays on the new mutants to evaluate the effect of the Arg103 substitutions on protein stability (Figure 3).

As seen with Arg103Asp (13), introduction of a negatively charged Glu residue at position

103 (Arg103Glu) also resulted in a protein that was significantly more resistant than wild-type Epo to heat denaturation (Fig.3B). Interestingly, the Asn substitution (Arg103Asn) also increased protein stability (Fig. 3A). The stability of Arg103His was similar to wild-type (Fig. 3D), whereas that of Arg103Gln (Fig. 3C) was reduced slightly and that of Arg103Lys (Fig. 3E) was reduced substantially. The stabilities of the positively charged mutants did not appear to follow a simple charge-based rule. The rank order of stabilities of the Arg103 substitutions was Asn, Asp, Glu>Arg, Ala, His  $\geq$  Gln > Lys

## DISCUSSION

In this study, we have introduced a series of single amino acid substitutions at position 103 of erythropoietin, in order to analyze the functional and structural requirements of this residue. Our results reveal that replacing Arg103 with Gln, Asn, Leu, or Glu generates a biologically inactive

molecule. Substitution with Lys or His, however, results in altered proteins with activities 25% and 2% that of wild-type Epo, respectively, suggesting a role for a positively charged (versus polar) residue at this position.

The pKa for the His side chain, in the absence of other local effects, is  $pK_a = 6.0$  (23). Under our assay conditions (pH 7.3), <5% of this moiety will be charged. This fact suggests that the activity measured for the Arg103His substitution mutant may be due to the residual charge present. However, a positively charged amino acid residue at position 103 is not sufficient to generate a fully functional erythropoietin molecule. A Lys side chain, which should be fully charged at pH 7.3, confers only 25% of wild-type activity upon the substitution mutant. Thus, either localized charge distributions or specific structural constraints necessitate the presence of Arg at this site for full activity. The introduction of various synthetic Arg analogs at position 103 of Epo would prove useful in addressing this issue (24).

Introduction of the negatively charged Glu at position 103 increased the protein's heat stability markedly. Previously, we obtained a similar result with another negatively charged substitution, Arg103Asp (13). These findings suggest the presence of a positive charge in close proximity to amino acid 103 which could interact with a negative charge placed at this position, thereby stabilizing the region. We tested the possibility that Asp (or Glu) substituted at position 103 might be interacting with nearby Lys97 or with Arg110. To accomplish this, we retained Arg103 and created Lys97Asp and Arg110Asp mutants, reasoning that interaction between a positively charged Arg103 and a negatively charged Asp97 or Asp110 should increase Epo's heat stability in a manner similar to that seen with the Arg103Asp and Arg103Glu mutants. However, Arg110Asp (36-46% activity of wild-type) showed no increase in heat stability, and Lys97Asp protein was not secreted in either of two independent transfections. In this regard, Wen et al. (14) have proposed that the side chain of Arg103 may exist in close proximity to that of Arg14. This hypothesis is consistent with the observed stability of our inactive mutants Arg103Asp and Arg103Glu. It also may imply that optimal activity requires a certain degree of charge repulsion between these two side chains. The reported reduced activity of Arg14 mutants supports this possibility. A systematic study of Arg (or Lys) to

Asp substitution mutants in this fashion should identify the relevant residue.

In assessing the function of arginyl residues in the structure and function of Epo it is important to note the unique structural/functional role of this amino acid in other proteins. White (25) analyzed 2775 protein sequences and determined the frequencies of each amino acid as a function of chain length. Notably, the frequencies of Arg and Lys are higher (and those of Asp and Glu are lower) in smaller proteins. He hypothesized that this represented the requirement of these smaller sequences for increased stabilization mediated via "increased hydrogen binding by Arg and increased hydrophobic stabilization due to the amphiphilic character of Arg and Lys." The frequencies of Arg and Lys in mature human Epo (165aa) are 7.2 and 4.8%, respectively, compared to ~5.5% and 5.5% respectively, for proteins of 160-170 aa analyzed by White. This relatively high frequency of Arg in Epo may be consistent with an intramolecular stabilizing role for some of the arginyls in the hormone. As Borders et al observe, Arg is capable of forming up to 4 H-bonds with adjacent carbonyls, and Lys can form 3 H-bonds (26). Alternatively, Arg103 of wild-type Epo (and Lys103 of Arg103Lys) may form intermolecular H-bonds with carbonyls of the Epo receptor, thus enhancing biological activity.

The observed specific activities of wild-type Epo (Arg 103) and mutant Arg103Lys are consistent with this interpretation. Thus, both the H-bonding and the positive charge of residue 103 may play a role in conferring sufficient flexibility to Epo's receptor binding domain, first, to facilitate the intermolecular interaction and, then, to contribute to stabilization of the Epo-Epo receptor binary complex.

The studies presented here emphasize the importance of Arg103 for the biological activity of Epo. Further characterization of this amino acid position by the methods noted above will greatly enhance our knowledge of Epo's structural and functional requirements for a productive Epo-Epo receptor interaction.

Table 1

Erythropoietin protein concentrations in transfected COS1 cell medium.

Construct	Erythropoietin Secreted (ng/ml*)
Wild-type	160
Arg103Aln	170
Arg103Asn	190
Arg103Asp	75
Arg103Gln	270
Arg103Glu	220
Arg103His	190
Arg103Lys	190

\*Determined by radioimmunoassay standardized against homogeneous recombinant human Epo.

Results are the means of replicate experiments. The range of values was equal to or less than 30%

of the mean for all samples.

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## FIGURE LEGENDS

Figure 1. Mutagenesis strategy for Arg103 of human erythropoietin. The nucleotide sequence corresponding to the portion of the wild-type Epo cDNA mutagenized is shown at the top. The nucleotides targeted by the mutagenic oligodeoxynucleotide are underlined. The specific nucleotide changes introduced to generate each listed mutant are depicted below the wild-type sequence. Unchanged nucleotides are depicted as a solid line.

Figure 2. Relative specific activities of the erythropoietin Arg103 mutants. Activities are expressed as a percentage of wild-type (Arg) erythropoietin. Results are the means of replicate experiments. The specific activities of wild-type recombinant erythropoietin ranged from 205-280 U/ $\mu$ g.

Figure 3. Comparison of heat stabilities of wild-type erythropoietin and Arg103 mutants. Wild-type, ( $\square$ ). (A), Arg103Asn ( $\blacksquare$ ). (B), Arg103Glu ( $\blacksquare$ ). (C), Arg103Gln ( $\blacksquare$ ). (D), Arg103His ( $\blacksquare$ ). (E), Arg103Lys ( $\blacksquare$ ).